Brain Pathology ISSN 1015–6305

RESEARCH ARTICLE

Fetal microchimerism in human brain tumors

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Keywords
brain tumor, fetal microchimerism, glioblastoma, meningioma, pregnancy.

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Received 15 April 2017
Accepted 13 September 2017
Published Online Article Accepted 18 September 2017

doi:10.1111/bpa.12557

Abstract

Sex differences in cancer incidence and survival, including central nervous system tumors, are well documented. Multiple mechanisms contribute to sex differences in health and disease. Recently, the presence of fetal-in-maternal microchimeric cells has been shown to have prognostic significance in breast and colorectal cancers. The frequency and potential role of these cells has not been investigated in brain tumors. We therefore selected two common primary adult brain tumors for this purpose: meningioma, which is sex hormone responsive and has a higher incidence in women, and glioblastoma, which is sex hormone independent and occurs more commonly in men. Quantitative PCR was used to detect the presence of male DNA in tumor samples from women with a positive history of male pregnancy and a diagnosis of either glioblastoma or meningioma. Fluorescence *in situ* hybridization for the X and Y chromosomes was used to verify the existence of intact male cells within tumor tissue. Fetal microchimerism was found in approximately 80% of glioblastoma cases and 50% of meningioma cases. No correlations were identified between the presence of microchimerism and commonly used clinical or molecular diagnostic features of disease. The impact of fetal microchimeric cells should be evaluated prospectively.

INTRODUCTION

Sex differences exist in the prevalence and prognosis of multiple tumor types (3, 11, 12, 30), including central nervous system tumors (45, 46). Multiple cell intrinsic and organismal mechanisms likely contribute to these sex differences. One potential difference between males and females is pregnancy. Parity has been associated with an alteration in cancer risk for a number of different tumor types (6, 9, 29, 38), and this association is not limited solely to cancers of the female reproductive system. During pregnancy, the female body undergoes dramatic changes to support the growth of the fetus, including developing a form of immunologic tolerance to prevent rejection of a genetically foreign organism (1, 24, 47). Remarkably, a requirement for active tolerance may not end with the birth of the child and may involve the persistence of fetal-in-maternal microchimeric cells.

During pregnancy, cells from the fetus traffic through the placenta into the mother. These cells can engraft and persist for decades (4, 34, 35), perhaps for life, resulting in a form of microchimerism. Fetal cells have been identified in tissues throughout the body, including the brain (8, 40), and appear to differentiate into a variety of different cell types (23, 40). The impact of these cells on maternal health is still under debate, but one area where they have been proposed to play a role is in cancer. The presence of microchimeric cells in blood has been prospectively associated with decreased risk for breast cancer and increased risk for colon cancer (22). In addition, microchimeric cells have been identified in tumors of the breast, lung, thyroid, cervix and skin (7, 10, 13, 32, 33, 35). In these studies, the frequency of microchimeric cells was increased in malignant neoplasms compared to surrounding normal tissue (10, 32, 35) and benign neoplasms (13, 33).

Although fetal cells have been previously identified in brain tissue (8, 40), whether these cells are present in tumors of the central nervous system has not yet been investigated. To address this question, we used quantitative PCR for the Y chromosome marker *DYS14* to look for the presence of male DNA in tumors from women with a diagnosis of either glioblastoma or meningioma and a history of male pregnancy. We then used fluorescence *in situ* hybridization against the X and Y chromosomes to verify the presence of intact male cells within tumor tissue. Although both male and female fetuses contribute to microchimerism, we focused on male cells because they provide the advantage of being easily identified by the presence of the Y chromosome.

Glioblastoma and meningioma are both common primary CNS tumors, but they differ in important ways. Notably, glioblastoma is slightly more common in men (36), while meningioma is more common in women (36, 49). Meningioma is also hormone responsive, with the majority of tumors expressing progesterone receptors (20, 42), while glioblastoma is not hormone responsive. Glioblastoma is intraparenchymal, heterogeneous and a World Health Organization Grade IV tumor (27), while meningioma is extra-axial, homogeneous and varies from Grades I–III (27). Given these distinguishing characteristics, determining the frequency of microchimeric cells in these two tumor types could provide insights into

whether distinct clinical features, or molecular mechanisms of oncogenesis, are associated with increased microchimerism.

Here, we show for the first time that fetal microchimeric cells are present within approximately 80% of glioblastoma and 50% of meningioma cases that occur in women with a history of male pregnancy. Women with glioblastoma were significantly more likely to be positive for male microchimerism. Within each tumor type, the frequency and abundance of fetal microchimeric cells did not correlate with distinguishing features in clinical history or molecular markers of oncogenic mechanisms. The impact of these cells on outcome will be important to prospectively assess, particularly as immunotherapy approaches to malignant brain tumors become more common.

MATERIALS AND METHODS

Patient selection

To facilitate the identification of microchimeric cells, we planned to use quantitative PCR and fluorescence *in situ* hybridization to detect the presence of the Y chromosome in tumors from female patients with either glioblastoma or meningioma, and positive pregnancy histories for male births. Medical records of female patients with a pathology diagnosis of either glioblastoma or meningioma at Barnes-Jewish Hospital in St. Louis, Missouri were reviewed in accordance with a Washington University Institutional Review Board (IRB)-approved Human Studies Protocol to identify women with a positive history of male pregnancy. A total of 205 cases were reviewed. Cases were included as positive if there was any reference to a male child in the medical record, regardless of whether a complete obstetric history was available. Thirty three positive cases were identified from women with a diagnosis of glioblastoma, and 26 positive cases were identified from women with a diagnosis of meningioma. Three samples from male patients were included as a control group. In addition to pregnancy history, charts were reviewed for age at tumor biopsy, histopathological diagnosis and WHO grade, tumor location and molecular findings such as IDH1 status and presence of EGFR amplification for glioblastoma cases, and progesterone receptor expression for meningioma cases. Normal brain parenchyma, when available based on review of H&E slides, was also evaluated for the presence of XY cells.

Isolation of DNA

Genomic DNA was isolated from formalin-fixed paraffin-embedded tissue sections using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany). DNA was isolated following the manufacturer instructions with minor modifications. Briefly, tissue sections were scraped off of glass slides using a clean razor blade and collected in a 1.5 ml microcentrifuge tube. Sections were deparafinnized by washing 3 times in xylene; following addition of xylene, samples were vortexed for 10 s, allowed to sit 10 minutes, then spun down. After the xylene washes, residual xylene was removed by washing 2 times with 100% ethanol and residual ethanol was allowed to evaporate. Samples were then resuspended in 180 μl buffer ATL, 20 μl proteinase K was added and samples were incubated for 1 h at 56°C, followed by 1 h at 90°C. To generate RNA-free genomic DNA, 5 μl of RNase A (10 mg/ml) was added and samples were incubated for 2 minutes at room temperature. Two hundred μl of buffer AL was then added, followed by 200 μl of 100% ethanol and samples were mixed by vortexing. The entire lysate was then transferred to the QIAamp MinElute column and spun down. Column was washed with buffer AW1, followed by buffer AW2, then dried by spinning at full speed for 3 minutes. To elute DNA, 35 μl of buffer ATE was added directly to the column, and incubated for 5 minutes, before spinning down at full speed for 1 minute. The eluted DNA was then reapplied to the column, and spun down a second time to further increase yield. DNA concentration and quality were assessed using a NanoDrop 1000 (Thermo Fisher, Waltham, MA, USA). All DNA isolation was performed by a female experimenter to prevent possible contamination of the samples with male DNA.

Quantitative PCR for DYS14

Quantitative PCR for the presence of male DNA was performed as described in Refs. (8, 52) with minor modifications. *DYS14*, a marker specific to the Y chromosome, was detected using Integrated DNA Technologies' Custom Probe-Based qPCR Assay (Integrated DNA Technologies, Coralville, IA, USA). DYS14 Probe sequence: CGA AGC CGA GCT GCC CAT CA (Dye/Quencher: FAM/ZEN/IBFQ); Forward Primer: CAT CCA GAG CGT CCC TGG; Reverse Primer: TTC CCC TTT GTT CCC CAA A. To quantify the frequency of Y positive cells present in each sample, a standard curve was established using purified male and female human genomic DNA (Promega, Fitchburg, WI). A dilution of 500, 100, 50, 10, 5, 1 or 0.5 cell equivalents of male DNA in a background of 20 000 cell equivalents of female DNA (conversion factor of 6.6 pg of DNA per cell) was performed to generate the standard. Purified female human genomic DNA was used as a control; controls were consistently negative. Between 4 and 6 replicates were run for each sample. A sample was considered positive if 50% or more of the replicates amplified. To control between samples for the relative number of cell equivalents of DNA loaded, qPCR for β-globin was performed on 2 replicates per sample and a standard curve was used to calculate cell equivalents of DNA. β-globin Probe sequence: AAG GTG AAC GTG GAT GAA GTT GGT GG (Dye/Quencher: FAM/ZEN/IBFQ); Forward Primer: GTG CAC CTG ACT CCT GAG GAG A; Reverse Primer: CCT TGA TAC CAA CCT GCC CAG. Mixed male and female genomic DNA was used to generate the standard curve consisting of 50 000, 25 000, 10 000, 5000, 1000, 500 and 100 cell equivalents of DNA. The square of the correlation coefficient for all standard curves was 0.95 or greater. Amplification conditions were an initial incubation at 95°C for 3 minutes to activate the polymerase, followed by 40 cycles of 95°C for 15 s and 60°C for 1 minute. A CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used to collect and analyze the amplification data. All qPCR was performed by a female experimenter to prevent possible contamination of the samples with male DNA.

Fluorescence in situ hybridization for X and Y chromosomes

Formalin-fixed paraffin-embedded tissue samples were cut to a thickness of 5 μm and mounted on positively charged slides. Slides were heated at 60°C for 40 minutes, then deparaffinized by washing in Citrisolve 3 times for 5 minutes each, followed by two washes in 100% ethanol for 1 minute each. Slides were air dried, then immersed in Pretreatment solution (Abbott Molecular, Abbott Park,

IL, USA) at 85°C for 20 minutes, rinsed in distilled water for 3 minutes, then immersed in Protease solution (Abbott Molecular, Abbott Park, IL, USA) at 37°C for 20 minutes. Slides were rinsed in distilled water for 3 minutes then air dried, followed by immersion in 70% ethanol, 85% ethanol and 100% ethanol for 1 minute each, then air dried again. Vysis CEP X SpectrumOrange/CEP Y SpectrumGreen direct labeled fluorescent DNA probe (Abbott Molecular, Abbott Park, IL, USA) was applied to the tissue, which was then coverslipped and codenatured by heating at 73°C for 5 minutes. Slides were hybridized overnight at 37°C in a humidified chamber. Slides were washed in 2xSSC/0.3% NP-40 at 74°C for 2 minutes, then 2xSSC/0.3% NP-40 at room temperature for 1 minute. Slides were counterstained with Vysis DAPI II Counterstain (Abbott Molecular, Abbott Park, IL, USA).

Cell counting

Using an Olympus BX61 fluorescent microscope (Olympus, Melville, NY, USA) with the Vysis DAPI/SpectrumOrange/SpectrumGreen filters and an 100x oil objective, 2000 cells were counted per slide and the number of X and Y chromosomes in each cell was recorded. Cells were counted if nuclei had good integrity, nuclear margins were clearly visible and chromosomal signals were distinct within the nucleus. Images were taken using the Jai Progressive Scan camera and CytoVision Imaging System (Leica Biosystems). Three glioblastoma cases were excluded from analysis due to insufficient quality of FISH staining for cell counting.

Hematoxylin & eosin

Hematoxylin & Eosin stain was performed using standard methods as a part of the initial diagnosis of cases.

EGFR amplification

For samples that did not have EGFR amplification status reported in the patient's chart, fluorescence *in situ* hybridization was performed to determine whether gene amplification was present. The same protocol as that used to detect the X and Y chromosomes was followed, with the following modifications. Slides were immersed in Pretreatment solution for 10 minutes, and immersed in Protease solution for 15 minutes. Slides were hybridized with the Vysis LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen probe (Abbott Molecular, Abbott Park, IL, USA). 200 cells were counted for each case, and a ratio of the average number of EGFR signals to the average number of CEP 7 signals was calculated.

IDH1 immunohistochemistry

For samples that did not have IDH1 status reported in the patient's chart, immunohistochemical staining was done to determine whether the $IDH1^{R132H}$ mutation was present. Staining was performed on BenchMark XT automated tissue staining systems (Ventana Medical Systems, Inc., Tucson, AZ, USA) using a validated protocol. Following blocking of endogenous peroxidase activity by hydrogen peroxide, and antigen retrieval using CC1 reagent (Ventana Medical Systems), tissue sections were incubated with mouse monoclonal anti-R132H-IDH1 (clone H09, Dianova, Hamburg, Germany) at 37°C for 20 minutes, followed by incubation with Ultra View HRP-conjugated multimer antibody reagent (Ventana Medical Systems). Antigen detection was performed using UltraView diaminobenzidine chromogen step (Ventana medical Systems), and sections were then counterstained with hematoxylin.

Statistics

All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). Fisher's exact test was used for comparing categorical data, and *t*-tests were used for comparing continuous data. All *t*-tests were two tailed.

RESULTS

Patient characteristics

A total of 205 charts were reviewed from women with a diagnosis of either glioblastoma or meningioma made at Washington University School of Medicine in St. Louis between 2013 and 2015. We excluded 98 women based on unclear or negative male pregnancy history and an additional 48 samples had insufficient tumor tissue for analysis. Two samples were excluded following review of the H&E stained sections; one was excluded due to insufficient viable tumor and a second was excluded because both tumor and normal brain were present in the section. In total 57 tumor samples were assessed for the presence of male DNA by quantitative PCR (qPCR): 32 glioblastoma cases and 25 meningioma cases. An additional three samples from male patients were included as a technical control (data not shown).

Patient characteristics are presented in Table 1. The average age at tumor resection did not significantly differ between glioblastoma patients and meningioma patients. The number of sons was also similar between glioblastoma patients and meningioma patients.

All the GBMs in our cohort were located supratentorially; three of these (9.4%) were positive for $IDH1^{R132H}$ by immunohistochemistry and were classified as secondary, and four (12.5%) were from clinically recurrent tumors. The majority of GBM's were solitary tumors; however, three women (9.4%) presented with multifocal disease. One woman had a coincident synchronous meningioma.

In contrast to the GBM samples, the meningioma cases showed greater variance in tumor location. While the majority of tumors were located in the convexities, nine samples (36.0%) were from tumors located in the skull base, and two (8.0%) were from tumors located in the spinal cord. Twenty meningiomas (80.0%) qualified as WHO Grade I, and the remaining five (20.0%) were classified as WHO Grade II, with one of these five showing evidence of brain invasion. The majority of meningiomas were primary, with only two samples (8.0%) from clinically recurrent tumors. Seven samples (28.0%) were from women with a history of multiple meningiomas, including one woman with meningeal meningiomatosis, and another with neurofibromatosis type 2.

Male DNA is present in human brain tumors

Following identification of women with a diagnosis of GBM or meningioma and a positive history of male pregnancy, a representative tissue block was chosen for each tumor. Genomic DNA was isolated from tissue sections, and quantitative PCR for the Y

Table 1. Patient characteristics.

	Glioblastoma (32)	Meningioma (25)
Age (years)		
Average	58.4 ± 12.4	54.8 ± 9.5
Median (Range)	59 (29–77)	52 (41–79)
Number of sons		
1	13 (40.6%)	7 (28.0%)
2	4 (12.5%)	2 (8.0%)
3	1 (3.1%)	1 (4.0%)
4	1 (3.1%)	0
5	1 (3.1%)	0
≥1	10 (31.3%)	11 (44.0%)
≥2	2 (6.3%)	4 (16.0%)
Location*		
Supratentorial/Convexities	32 (100%)	14 (56.0%)
Skull base	0	9 (36.0%)
Spinal cord	0	2 (8.0%)
	*GBM: Supratentorial, spinal cord, Meningioma: Convexities, skull base, spinal cord	
WHO Grade*		
I	0	20 (80.0%)
II: with brain invasion	0	5 (20.0%): 1
III	0	0
IV	32 (100%)	0
	*Meningioma: WHO Grades I–III, GBM: WHO Grade IV	
Primary, Secondary, or Recurrent		
Primary	25 (78.1%)	23 (92.0%)
Secondary	3 (9.4%)	0
Recurrent	4 (12.5%)	2 (8.0%)
Solitary or Multifocal/Multiple		
Solitary	29 (90.6%)	18* (72.0%)
Multifocal/Multiple	3* (9.4%)	7† (28.0%)
	*1 case with synchronous multifocal GBM and meningioma. †1 case of meningeal meningiomatosis.	

chromosome marker *DYS14* was performed. Between 4 and 6 replicates were run for each sample, and samples were considered positive if 50% or more of the replicates showed amplification.

In women with a positive history of male pregnancy, we were able to identify the presence of male DNA in both glioblastoma and meningioma samples. In the glioblastoma cases, 25 of the 32 samples (78.1%) were positive (Table 2). In the meningioma cases, 12 of the 25 samples (48.0%) were positive (Table 2). The prevalence of male microchimerism was significantly higher in the glioblastoma cases than in the meningioma cases ($P < 0.05$, Fisher's exact test).

To extrapolate the frequency of male cells within an individual sample, we used a standard curve to determine the relative genomic equivalents of male DNA present. We normalized to the total genomic equivalents of DNA loaded for each sample, then calculated the number of Y positive cells present in 100 000 female cells. In the glioblastoma samples, the frequency of Y positive cells ranged from <1 to 46.1 in 100 000, with an average of 4.81 Y positive cells in 100 000 (Figure 1; Table 2). In the meningioma samples, the frequency of Y positive cells ranged from <1 to 7.3 in 100 000, with an average of 2.05 Y positive cells in 100 000 (Figure 1; Table 2). Although the frequency of male cells appeared

Table 2. Prevalence of male microchimerism in human brain tumors (by qPCR).

	Y Positive	Y Negative	Range*	Mean*†	Median*†	90th percentile*†
Glioblastoma	25/32 (78.1%)	7/32 (21.9%)	0–46.14	4.81	1.14	12.13
Meningioma	12/25 (48.0%)	13/25 (52.0%)	0–7.27	2.05	0.83	4.45

*Normalized frequency of Y positive cells per 100 000 female cells.

†Positive samples only.

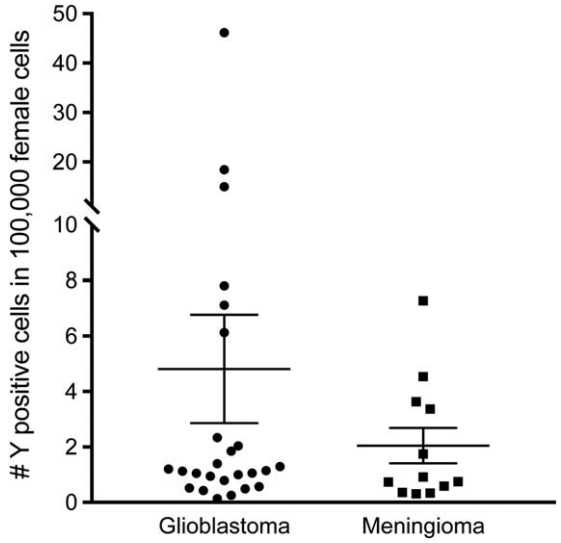

Figure 1. *Male DNA is present in human brain tumors.* Normalized frequency of Y positive cells in 100 000 female cells as determined by quantitative PCR for the Y chromosome marker *DYS14*. Each dot depicts results from a representative tissue block from an individual woman with a diagnosis of either glioblastoma or meningioma and a positive history of male pregnancy. Only samples that were determined to be positive for fetal microchimerism based on amplification of 50% or more of qPCR replicates are plotted (glioblastoma $n = 25$; meningioma $n = 12$).

to be greater in the positive glioblastoma samples than in the positive meningioma samples, this difference was not significant.

Thus, male DNA was identified in two different types of human brain tumors with a prevalence of 50–80%. It is likely that a high proportion of women with brain tumors have fetal microchimeric cells within the tumor microenvironment.

Intact male cells are present in human brain tumors

Although quantitative PCR is a highly sensitive technique, it is not able to distinguish between free genomic DNA and the presence of intact fetal cells. Fluorescence *in situ* hybridization, while less sensitive, provides the ability to visualize intact cells and ensure that cells are located within the tumor tissue itself.

To verify that the male DNA identified in tumor sections corresponds to intact male cells, we performed fluorescence *in situ* hybridization against the X (Xp11.1-q11.1 Alpha Satellite DNA) and Y (Yq12; Satellite III region) chromosomes, on tissue sections from samples positive by PCR (Figure 2). 2000 cells were counted for each sample, and the number of Y positive cells was recorded; 3 GBM samples were excluded based on insufficient technical quality of the FISH. In 3 of 22 positive GBM samples and in 2 of 12 positive meningioma samples, rare male cells were identified by FISH. All male cells had intact nuclei, and were located within the bulk tumor as identified by H&E.

Thus, we observed intact fetal cells in both glioblastoma and meningioma samples. Although XY cells were only seen in a few samples by FISH, this is likely a result of the rare nature of these cells, and the limited number of total cells counted. Based on these findings, it seems likely that the male DNA observed in other tumor samples also corresponds to the presence of intact fetal microchimeric cells.

Y positive cells are present in histologically normal brain

Given results of previous studies showing increased fetal microchimerism in tumors compared to surrounding normal tissue (10, 32, 35), we wondered whether microchimerism might be similarly increased in brain tumors compared to normal brain. We identified slides that contained brain parenchyma without signs of tumor involvement and used these to assess whether microchimerism prevalence differs in surrounding, apparently normal, tissues. Because of the highly invasive nature of GBM, and the challenges of distinguishing normal cells from infiltrating tumor cells, we limited this identification to patients with meningiomas.

We identified four women with slides containing histologically normal brain without evidence of tumor involvement. We performed qPCR on these tissue sections and compared the frequency of Y positive cells to the frequency of Y positive cells in the corresponding meningioma section (Figure 3A). Surprisingly, there was a trend towards increased numbers of male cells in the histologically normal brain samples ($P = 0.09$, paired *t*-test). We next compared the frequency of Y cells in the normal brain sections to the frequency of Y cells in all meningioma and glioblastoma samples (Figure 3B). When compared to all meningioma samples, the frequency of Y positive cells was not increased in normal brain ($P = 0.95$; *t*-test) Although the frequency of male cells appeared to be higher in the glioblastoma samples, this did not reach statistical significance.

To confirm that the male DNA observed in the histologically normal brain corresponds to the presence of intact male cells, we performed FISH for the X and Y chromosomes on tissue sections from the four women above, as well as for a fifth woman that was excluded from the PCR studies because she had areas of histologically normal brain and tumor on the same tissue section. To improve our ability to detect Y positive cells, we increased the number of cells counted to 4000.

Intact male cells were observed in three of the sections containing tumor and in two of the sections containing histologically normal brain (Table 3). Surprisingly, intact male cells were observed for one tumor sample that fell below the cut-off of positivity by PCR (33% of wells amplified); suggesting that our conservative criterion may be underestimating the true number of women with fetal microchimerism.

Presence of fetal microchimerism did not correlate with specific clinical or molecular features of disease within each tumor type

Clinical characteristics of the women positive for male microchimerism are presented in Tables 4 and 5. Although our sample size was relatively small, we compared these clinical and molecular characteristics to determine if there were any clear correlations with microchimerism. Neither the presence nor frequency of microchimeric cells appeared to correlate with number of sons or with the age of the patient. Microchimerism was found in women with as few as 1 son and as many as 5, and ranging in age from 29 to 79.

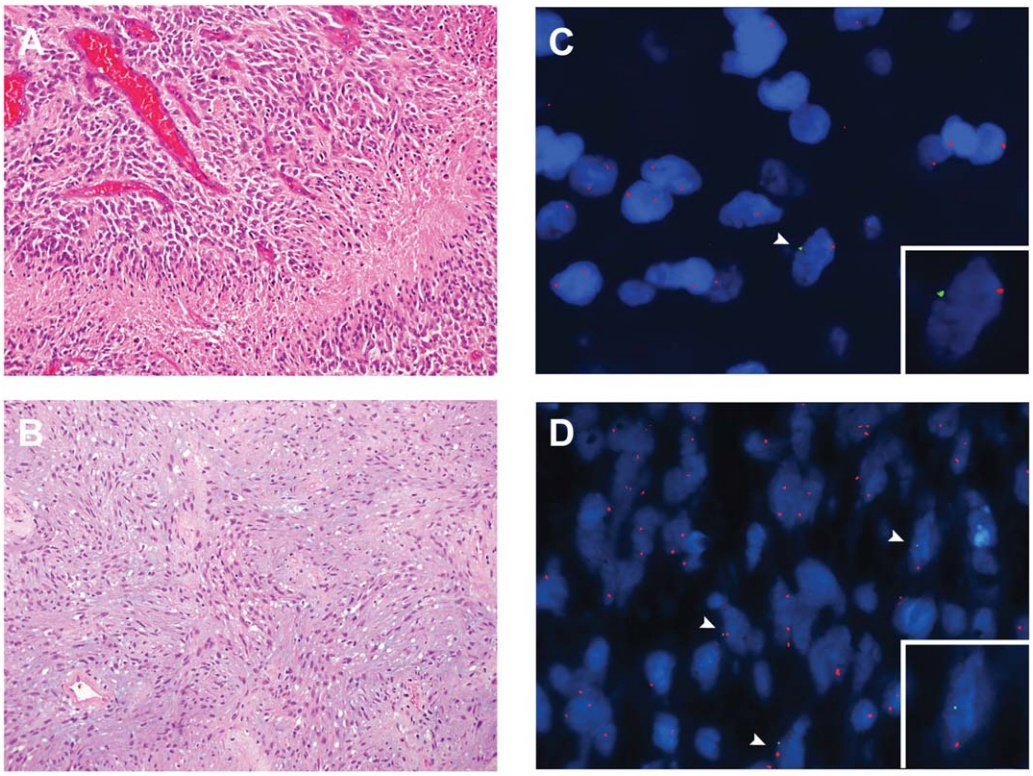

Figure 2. *Fetal microchimerism is present in human brain tumors.* **A, B.** Representative images of H&E stained slides from a glioblastoma (A) and meningioma (B) taken at 20x magnification. **C, D.** Representative images of X and Y chromosome FISH results in a glioblastoma (C) and meningioma (D) taken at 100x magnification. Arrowheads indicate the presence of XY cells. Lower right insets show a magnified version of a representative XY cell.

Within the glioblastoma samples there was no clear correlation between the presence or frequency of microchimeric cells and common clinical and molecular characteristics of disease (Table 4). Positive samples included primary, secondary and recurrent GBMs, as well as both solitary and multifocal tumors. Positive samples were also mixed in regards to MGMT methylation (positive methylation is associated with improved response to chemotherapy), EGFR amplification (a common genetic alteration associated with primary GBM), and $IDH1^{R132H}$ status (a marker of secondary GBM associated with improved prognosis).

Within the meningioma samples positive for microchimerism there was a similarly large degree of clinical and molecular variability (Table 5). Positive samples included tumors located both supratentorially and in the skull base, and of WHO Grades I and II. While most of the positive tumors were primary, one was recurrent, and samples came from women with both solitary and multiple meningiomas. Progesterone receptor staining ranged from widespread to absent. There were also a number of meningioma variants represented, including atypical meningioma, chordoid meningioma and angiomatous meningioma.

Presence of fetal microchimerism and survival in glioblastoma patients

To address whether fetal microchimerism might be associated with improved outcome in disease, we compared survival time in women with glioblastoma who were positive or negative for fetal microchimerism. This analysis was limited to glioblastoma patients, since meningioma is commonly a benign disease with prolonged survival. We were able to ascertain survival time for 26 of the 32 glioblastoma patients for which we had qPCR results. Average survival time was 1.97 years in the women who were positive for fetal microchimerism and 0.97 years in the women who were negative for fetal microchimerism (Figure 4; $P = 0.16$, *t*-test). Although this difference did not reach statistical significance, potentially due to our small sample size, the finding is intriguing and suggests a potential protective role for fetal microchimerism in GBM.

DISCUSSION

The presence of fetal-in-maternal microchimeric cells has only recently been recognized as a potential factor in cancer susceptibility for women. Here, we show that fetal cells can be found in two different types of human brain tumors at a prevalence of approximately 50–80%. This is the first study to identify fetal microchimerism within tumors of the CNS. We used a fairly conservative measure to define positive cases by qPCR, requiring that at least 50% of replicates undergo amplification. In addition, because our methods were limited to the detection of male cells, we were unable to identify fetal cells originating from female pregnancies; thus, our results may be underestimating the true prevalence of microchimerism in women with brain tumors. Regardless, our findings indicate

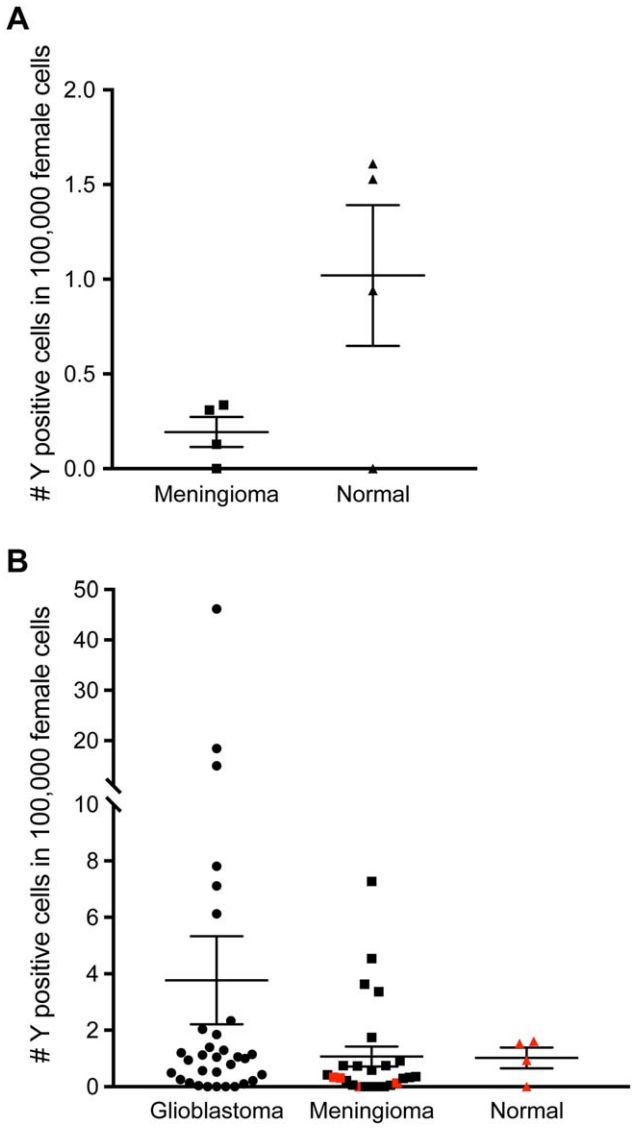

Figure 3. *Frequency of male cells in glioblastoma, meningioma and normal brain samples*. **A**. Normalized frequency of Y positive cells in 100 000 female cells as determined by quantitative PCR for the Y chromosome marker *DYS14*. Samples are from four women with matching tissue sections that showed either meningioma or histologically normal brain without tumor based on H&E. ($P = 0.09$; paired *t*-test, $n = 4$). **B**. Normalized frequency of Y positive cells in 100 000 female cells as determined by quantitative PCR for the Y chromosome marker *DYS14* in all glioblastoma ($n = 32$), meningioma ($n = 25$) and normal brain samples ($n = 4$). Samples highlighted in red correspond to the samples graphed in (A).

that a high proportion of women with brain tumors have evidence of allogeneic cells within their tumor.

The frequency of microchimeric cells was rare, ranging from <1 cell per 100 000 to 46 cells per 100 000. Although we believe this represents a measure of the relative frequency between samples, this may not be a true quantitation of the number of male cells present. We are working with FFPE samples, which often have highly degraded and fragmented DNA. To compensate for this, our PCR amplicon is short (<150 bp), but we may still not be able to detect all copies of the Y chromosome present. Additionally, GBMs have frequent chromosomal aberrations, including polyploidy; since we normalize to genome equivalents of DNA, our calculated frequency does not account for this, and consequently we may be underestimating the number of Y cells present.

A possible limitation of PCR is the potential for contamination and thus false positives. We have tried to minimize this risk by imposing a conservative definition of positivity, requiring that 50% or more of replicates amplify. We have also decreased our risk of contamination by having all DNA isolation and qPCR studies performed by a female experimenter. Finally, we ran purified human female genomic DNA on all plates as a negative control, and these controls were consistently negative. Nevertheless, it is possible that some of the women identified as positive by PCR could in fact be false positives, and that we may be over estimating the prevalence of fetal microchimerism. However, the visualization of intact male cells by FISH provides confirmation that fetal microchimeric cells can be present in brain tumors in women, and the PCR results suggests that the percentage of women for which this is true may be quite high.

Another limitation of our study is that our methods are not specific solely to the detection of fetal cells, but instead detect the presence of any male DNA. While the most common acquisition of male cells is through pregnancy with a male fetus, there are other potential means by which a woman can acquire male DNA, including transfer of cells from a male twin or older brother, or through blood transfusion (31, 52). Because pregnancy is the most common source of exposure to allogeneic cells, we focused our studies on women with a history of male pregnancy, but these other sources of microchimerism have the potential to impact women without pregnancies and even males as well; this is likely to be far less common, however.

Our study did not identify any specific clinical or molecular features within an individual tumor type that were associated with microchimerism. However, we did find that samples from women with glioblastoma were significantly more likely to be positive for fetal microchimerism than those from women with meningioma. Additionally, the average frequency of male cells was approximately 3 times higher in the glioblastoma samples than in the meningioma and normal brain samples, although this difference did not reach statistical significance. There are a few potential explanations for this finding. (1) Fetal microchimeric cells are more likely to accumulate within glioblastomas, leading to an increased ability to detect microchimerism. (2) Fetal microchimeric cells are excluded from meningiomas, leading to a decreased ability to detect microchimerism. (3) Women with fetal microchimerism have an increased risk of developing glioblastoma. Regardless, these findings suggest that the presence of fetal microchimerism in brain tumors is unlikely to result purely from background levels of microchimerism in the normal brain, since we expect this to be equivalent across all groups.

The two tumor types investigated were chosen because they differed in a number of key characteristics. Meningioma is typically a low-grade tumor, and all meningiomas in our study were WHO Grade I or II. In contrast, GBM is a high-grade, and highly malignant tumor. Our finding that glioblastoma samples have higher levels of microchimerism raises intriguing questions about whether this might be dependent on tumor grade. In future studies, it would

Table 3. Presence of male microchimerism in adjacent normal brain parenchyma.

Case ID	Tumor Positive or Negative by qPCR	Adjacent brain Positive or Negative by qPCR	Tumor # Y+ Cells FISH (4000 count)	Adjacent brain # Y+ Cells FISH (4000 count)
WU-M2	Positive	Negative	0	0
WU-M9	N/A	N/A	4	2
WU-M12	Negative	Negative	3	0
WU-M13	Negative	Positive	0	0
WU-M16/WU-G20	Positive	Positive	1	3

be interesting to include samples from patients with low grade gliomas to address how the differences in microchimerism prevalence extend to a tumor of similar cellular origin but lower grade and malignancy.

It is also intriguing to speculate about the origin of the fetal microchimeric cells within the brain tumor samples. Meningiomas are extra-axial tumors, while glioblastomas are intraparenchymal tumors. Nonetheless, fetal cells appear to be capable of infiltrating both tumor types, and are found at even higher levels within GBMs. Blood brain barrier dysfunction is known to occur in GBM (26, 43, 51), and it is possible that this contributes to the ability of fetal microchimeric cells to traffic into the tumor. An additional possibility is that the fetal cells identified may have already been present in the maternal brain prior to tumor formation. The normal brain is known to harbor microchimeric cells (8, 40), and we identified male cells in samples of normal brain from meningioma patients. Thus, these cells could potentially either migrate within the brain or the meninges to tumor sites or be surrounded by the tumor as it grows.

Another significant difference between meningioma and GBM is that meningioma is more common in females, while GBM is more common in males. Meningioma occurs with a peak female to male ratio of approximately 3:1 in women ages 35–44 (49), while GBM is approximately 1.6 times more common in men than women (36). Prior studies have examined whether parity is associated with an alteration in cancer risk for brain tumors; however, for both glioblastoma and meningioma the results are conflicting. While some studies found a decrease in risk associated with parity and increased number of pregnancies for gliomas (6, 19, 25, 50), others found no difference (2, 21, 28, 44). Studies in meningioma are equally varied (49), although

Table 4. Clinical and molecular characteristics of tumors from women with glioblastoma and positive male fetal microchimerism.

Case ID	# Y+ cells (qPCR)	Age	# of sons	Tumor location	Pathology diagnosis	WHO grade	Primary, secondary or recurrent	Solitary or multifocal	MGMT methylation	EGFR amplification	IDH1 (R132H)
WU-G30	46.138	56	2	Supratentorial	Glioblastoma	IV	Secondary	Solitary	Positive	Negative	Positive
WU-G34	18.442	51	1	Supratentorial	Glioblastoma	IV	Primary	Solitary	Negative	Negative	Negative
WU-G14	15.008	57	2	Supratentorial	Glioblastoma	IV	Primary	Solitary	Negative	Positive	Negative
WU-G7	7.811	69	1	Supratentorial	Glioblastoma	IV	Primary	Solitary	Positive	Negative	Negative
WU-G8	7.114	63	1	Supratentorial	Glioblastoma	IV	Primary	Multifocal	Negative	Negative	Negative
WU-G3	6.128	77	5	Supratentorial	Glioblastoma	IV	Primary	Solitary	Negative	Negative	Negative
WU-G4	2.333	39	1	Supratentorial	Glioblastoma	IV	Recurrent	Multifocal	Not available	Negative	Not available
WU-G13	2.036	52	4	Supratentorial	Glioblastoma	IV	Primary	Solitary	Positive	Not informative	Negative
WU-G20	1.851	67	1	Supratentorial	Glioblastoma	IV	Primary	Multifocal*	Not available	Negative	Negative
WU-G6	1.393	56	≥1	Supratentorial	Glioblastoma	IV	Primary	Solitary	Negative	Negative	Negative
WU-G27	1.292	75	2	Supratentorial	Glioblastoma	IV	Primary	Solitary	Positive	Positive	Negative
WU-G23	1.202	74	≥2	Supratentorial	Glioblastoma	IV	Primary	Solitary	Negative	Negative	Negative
WU-G25	1.144	60	≥1	Supratentorial	Glioblastoma	IV	Primary	Solitary	Negative	Negative	Negative
WU-G2	1.127	58	1	Supratentorial	Glioblastoma	IV	Primary	Solitary	Negative	Negative	Negative
WU-G21	1.057	31	1	Supratentorial	Glioblastoma	IV	Secondary	Solitary	Positive	Negative	Positive
WU-G19	1.049	40	1	Supratentorial	Glioblastoma	IV	Secondary	Solitary	Negative	Negative	Positive
WU-G17	0.998	29	1	Supratentorial	Glioblastoma	IV	Primary	Solitary	Negative	Negative	Negative
WU-G5	0.943	73	1	Supratentorial	Glioblastoma	IV	Primary	Solitary	Not available	Not informative	Negative
WU-G10	0.788	56	≥1	Supratentorial	Glioblastoma	IV	Primary	Solitary	Negative	Negative	Negative
WU-G9	0.568	65	≥2	Supratentorial	Glioblastoma	IV	Recurrent	Solitary	Negative	Not informative	Negative
WU-G15	0.517	53	≥1	Supratentorial	Glioblastoma	IV	Primary	Solitary	Positive	Negative	Negative
WU-G33	0.492	46	1	Supratentorial	Glioblastoma	IV	Primary	Solitary	Positive	Negative	Negative
WU-G32	0.426	61	≥1	Supratentorial	Glioblastoma	IV	Primary	Solitary	Not available	Negative	Negative
WU-G29	0.257	72	≥1	Supratentorial	Glioblastoma	IV	Primary	Solitary	Positive	Positive	Negative
WU-G24	0.137	62	≥1	Supratentorial	Glioblastoma	IV	Recurrent	Solitary	Positive	Positive	Negative

*Coincident Solitary Meningioma (WU-M16).

Table 5. Clinical and molecular characteristics of tumors from women with meningioma and positive male fetal microchimerism.

Case ID	# Y+ cells (qPCR)	Age	# of sons	Tumor location	Pathology diagnosis	WHO grade	Primary or recurrent	Solitary or multiple	Progesterone receptor
WU-M10	7.272	56	≥1	Convexity	Meningioma with worrisome features	I	Primary	Multiple	Positive, focal
WU-M15	4.536	52	≥1	Convexity	Meningioma	I	Primary	Solitary	Positive, widespread
WU-M23	3.632	51	≥1	Skull base	Meningioma with extensive osseous metaplasia	I	Primary	Solitary	Positive, widespread
WU-M29	3.368	57	1	Convexity	Meningioma	I	Primary	Solitary	Negative
WU-M3	1.744	53	1	Skull base	Meningioma	I	Primary	Solitary	Positive
WU-M32	0.915	44	≥1	Convexity	Meningioma	I	Primary	Solitary	Positive, widespread
WU-M20	0.747	52	≥1	Skull base	Meningioma	I	Primary	Solitary	Not available
WU-M33	0.734	42	1	Convexity	Meningioma	I	Primary	Multiple	Positive, focal
WU-M19	0.586	49	≥2	Skull base	Meningioma	I	Primary	Solitary	Positive, widespread
WU-M6	0.356	79	≥1	Convexity	Atypical meningioma with focally increased proliferation index	II	Primary	Multiple	Positive, widespread
WU-M16	0.336	67	1	Convexity	Meningioma, angiomatous variant	I	Primary	Solitary*	Positive, focal
WU-M2	0.309	50	≥1	Skull base	Chordoid meningioma with brain invasion	II	Recurrent	Solitary	Positive, widespread

*Coincident Multifocal GBM (WU-G20).

a recent meta-analysis did find a borderline significant increase in meningioma risk for parous women compared to nulliparous women (39). A lack of consistency in the association between parity and cancer risk does not exclude a possible contribution of microchimerism. There is little consistency between numbers of sons and presence of fetal microchimerism in blood (22), and even less is known about what the correlation is with levels in brain. In addition, spontaneous abortions have been shown to contribute to microchimerism (37, 52). Thus, studies examining parity may be missing a number of women whose brains contain fetal cells, potentially obscuring a relationship between pregnancy and cancer risk. Whether the presence of fetal microchimerism is associated with a change in the risk of developing either meningioma or glioblastoma is something that will need to be addressed in future prospective studies.

The low frequency of Y positive cells detected in our samples indicates that fetal cells do not contribute to the neoplastic clone(s), but are instead most likely a part of the tumor stroma. This finding is consistent with prior studies of fetal microchimerism in solid tumors, which found a similarly low abundance of XY cells (7, 10, 13, 33). The importance of the tumor stroma to brain tumor biology is now well established (5, 15, 17, 41). Our finding that fetal allogenic cells are an additional component of this stroma in a high percentage of women is a novel and exciting one. Although these cells are rare, the relevance of stromal density to tumor biology is not yet well defined, and it is possible that even very rare stromal cell populations have biological relevance. Previous studies have reported levels of microchimerism as low as 2 per 1 million genomes and found an association with cancer risk (22), and the level of frequency we observed in our brain tumor samples is similar to that seen in prior studies of fetal microchimerism in breast cancer samples (13, 16). Our finding that women with glioblastoma who were positive for microchimerism had a longer average survival time than women who were negative for microchimerism, while not significant, raises the intriguing possibility that fetal microchimeric cells may influence disease outcome in GBM, potentially acting in a protective capacity. Future prospective studies with increased patient numbers are needed to better determine

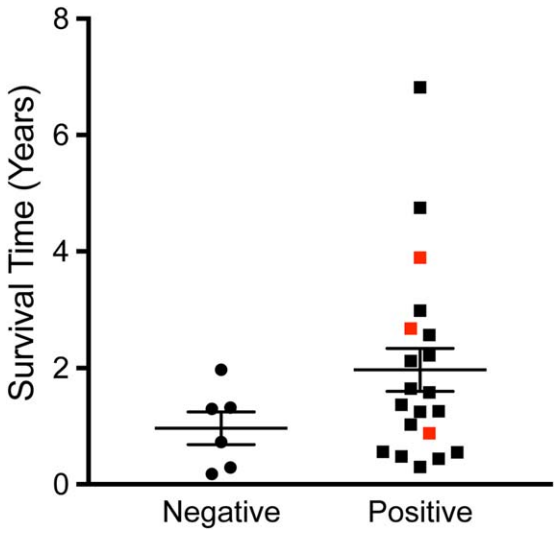

Figure 4. *Survival time in glioblastoma patients with and without fetal microchimerism.* Graph of individual survival times for women determined to be either positive ($n = 20$) or negative ($n = 6$) for fetal microchimerism by quantitative PCR for the Y chromosome marker *DYS14* ($P = 0.16$; *t*-test). Samples highlighted in red correspond to cases of secondary GBM, which is generally associated with improved survival time.

the health implications of fetal microchimerism in both glioblastoma and meningioma.

The mechanism by which fetal microchimeric cells may contribute to cancer risk and outcome is not yet well understood. A limited number of studies have performed combined FISH and immunofluorescence in tumor specimens, and results thus far suggest that fetal cells can potentially take on multiple phenotypes within the tumor microenvironment. In breast carcinoma samples, for example, 22% of XY cells expressed cytokeratin, an epithelial marker and 16% expressed vimentin, a mesenchymal marker, while none of the XY cells were positive for CD45, a leukocyte marker (13). In cervical cancer samples in contrast, 44% of XY cells were positive for CD45, and 24% were positive for cytokeratin (7). In samples from papillary thyroid cancer, 13% of XY cells were positive for CD45, and 66% were positive for thyroglobulin, a thyrocyte marker (10). Finally, in human melanoma samples 71% of XY cells were positive for the endothelial markers CD34 or CD31, and 20% were positive for CD45 (33). While some studies have proposed a beneficial role for microchimeric cells, possibly though promoting or contributing to tissue repair, or as natural killer cells or cytotoxic T cells targeting cancer cells, other studies have postulated that they play a harmful role by contributing to tumor angiogenesis. In future studies, it will be important to determine what phenotype microchimeric cells adopt within both the tumor environment and the normal brain; this may provide further insight into their function in brain tumor biology. Regardless of the form and function they may take, microchimeric cells at their most basic level represent a type of allogeneic transplant. Their persistence in maternal tissues suggests that mechanisms of immune tolerance or suppression may be active in the mother. The interplay of fetal microchimeric cells and the maternal immune system appears to be complex, and is still being actively investigated. Given recent interest in applying cancer immunotherapy to brain tumors (14, 18, 48), it might be potentially important to consider whether the presence of microchimerism impacts treatment efficacy and side effects of this therapy type. It may be necessary to study immunotherapy in men and women separately, or perhaps even more specifically in women with and without evidence of fetal microchimerism in their tumor resection.

ACKNOWLEDGMENTS

We thank the Washington University School of Medicine Clinical and Molecular Cytogenetics Laboratory for their assistance with FISH and cell counting. This work was supported by the McDonnell Center for Cellular and Molecular Neurobiology (JBR, SD). The authors declare no conflict of interest. This study was presented in part at the annual Society of Neuro-Oncology meeting held in Scottsdale, Arizona, on November 19, 2016 (abstract #2704).

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